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Rapid spectrophotometric and fluorimetric methods for monitoring nitrogenous (proteinaceous) compounds in cheese and cheese fractions: a review

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This article reviews various techniques which could be used to quantify nitrogenous compounds (proteins, peptides and/or free amino acids) in cheese. Chemical (macro-Kjeldahl), spectrophotometric [ultra-violet, Hull, Lowry, dye-binding (Amido Black 10B, Bradford, erythrosin), ninhydrin, trinitrobenzene-sulphonic acid and *o*-phthaldialdehyde (OPA)] and fluorometric (OPA and fluorescamine) procedures are discussed. Many of these techniques are commonly used in cheese analysis, while others have not been applied to cheese.

INTRODUCTION

The formation of water-soluble nitrogen in Cheddar cheese during ripening indicates the extent of proteolysis (Bondzynski, 1894; Kuchroo and Fox, 1982*a*; Fox, 1989; Gonzalez de Llano *et al.*, 1993). The flavour, texture and formation of flavour precursors is greatly affected by the rate and extent of protein degradation (Adda *et al.*, 1982). Numerous techniques, based on the structure of proteins, peptides and/or amino acids, have been developed to quantify nitrogen compounds in food systems such as cheese, in the presence of other food constituents (fat, carbohydrates, vitamins and minerals). The majority of these techniques are spectrophotometric or fluorimetric; as turbidity interferes with these assay techniques, their use is limited to soluble nitrogenous compounds.

A number of fractionation schemes have been used to extract soluble nitrogenous compounds from cheese (as reviewed by Fox *et al.*, 1995). Cheese peptides have been solubilised in urea (McSweeney *et al.*, 1993), water (Kuchroo and Fox, 1982*a*) or buffers at pH 4.6 (Kuchroo and Fox, 1982*a*; Addeo *et al.*, 1992), $CaCl_2$ (Venema *et al.*, 1987), $BaCl_2$ (Petersen *et al.*, 1957), chlorofom/methanol (Harwalkar and Elliott, 1971; Smith and Nakai, 1990), butan-1-ol (Motoba *et al.*, 1970), ethanol (Kuchroo and Fox, 1982*a*; Christensen *et al.*, 1991), phosphotungstic acid (Jarrett *et al.*, 1982), sulphosalicilic acid (Cliffe and Law, 1991), picric acid (Reville and Fox, 1978), $Ba(OH)_2$-$ZnSO_4$ (Hickey *et al.*, 1983) or EDTA (Kuchroo and Fox, 1982*b*). These methods of peptide fractionation were described in detail in recent reviews (Fox *et al.*, 1995; Fox and McSweeney, 1996).

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PROTEIN OR PEPTIDE QUANTITATION

Kjeldahl procedure

The most commonly used method for quantifying nitrogen in cheese and fractions thereof is the Kjeldahl procedure (IDF Standard, 1964). It has been used extensively to quantify total nitrogen in cheese (Phelan *et al.*, 1973; Noomen, 1977; O'Keeffe *et al.*, 1978; Banks, 1988) and cheese analogues (Mulvihill and McCarthy, 1993), water-soluble extracts (Visser, 1977; Reville and Fox, 1978; Aston *et al.*, 1983*a*,*b*; O'Sullivan and Fox, 1990), the non-protein nitrogen fraction of cheese (Visser, 1976) and 5% phosphotungstic acid (PTA)-soluble cheese extracts (Jarrett *et al.*, 1982). The method is based on nitrogen determination and involves an acid digestion step during which organic nitrogen is converted to nitrogen in the form of non-volatile ammonium sulphate. The digest is made strongly alkaline, the ammonia distilled off, trapped in acid and quantified by

acid–base titration. Since milk protein is presumed to contain approximatly 15.67% N, a factor of 6.38 is used to convert total nitrogen to total protein in milk and milk products.

The Kjeldahl procedure is highly repeatable, and, unlike spectrophotometric and fluorimetric methods, is suitable for quantitation of nitrogen compounds (proteins, peptides, amino acids, amines, ammonia) in solid, semi-solid or turbid samples. However, phospholipids, nucleic acids and amino sugars, which contain low levels of nitrogen, may contribute to the final results. The principal disadvantage of using this method, however, is that it is tedious and time-consuming, and although the method has been automated, exposure to dangerous and corrosive chemicals is unavoidable. Therefore, there is a growing demand for rapid, safe methods for quantifying nitrogen compounds in cheese and cheese extracts (Ardo and Meisel, 1991; Fox *et al.*, 1995).

Ultra-violet spectrophotometric method

Many protein assay techniques depend on the presence of specific amino acids in the protein. The ultra-violet (UV) method of Vakaleris and Price (1958) is based on the absorbance of amino acids containing an aromatic functional group (tyrosine and/or tryptophan) at ~280 nm. The UV method for protein determination is not widely used to measure the level of water-soluble peptides in cheese but is commonly used to quantify protein in eluates from chromatographic columns (Singh *et al.*, 1994; McSweeney *et al.*, 1994). Vakaleris and Price (1958) measured pH 4.6-soluble peptides by this method as an index of proteolysis in cheese. The concentrations of tryptophan and tyrosine have been used as an index of β-casein degradation in cheese (Marcos and Esteban, 1993). When α_{s1}-casein is hydrolysed early in cheese ripening, N-terminal fragments, containing no Tyr or Trp are produced. These peptides do not absorb UV radiation but on hydrolysis of β-casein later during ripening, the peptides released contain Tyr (but no Trp). Therefore, the degradation of β-casein in cheese can be monitored by UV absorbance (Marcos and Esteban, 1993). The UV method is not, however, generally used to monitor degradation of β-casein in cheese.

Hull procedure

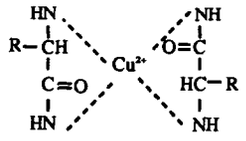
The procedure of Hull (1947) also depends on the presence of tyrosine which reacts with the Folin-Ciocalteau reagent, forming a blue-coloured complex. The method was modified by Citti *et al.* (1963) and has since been used to monitor TCA-soluble nitrogen in milk (Arnott *et al.*, 1957; Morris *et al.*, 1961; Dulley and Kitchen, 1972; Martley and Lawrence, 1972; Lin *et al.*, 1982; Samples *et al.*, 1984). The Hull method lacks sensitivity and is cumbersome to use with large numbers of samples (Church *et al.*, 1983). Lin *et al.* (1982) reported that the Hull procedure was more accurate than the UV method as a measure of soluble tyrosine in blue cheese because the latter was subject to interference from other compounds in the extracts.

Lowry method

Similar to the Hull procedure, the Lowry method is partially dependent on the reaction of tyrosine and tryptophan with the Folin-Ciocalteau reagent. The Lowry method is, however, more complicated as it involves the reaction of copper with peptide bonds in alkaline solution (Biuret reaction). The combination of these two reactions results in the development of a blue colour, the intensity of which can be determined at 750 nm (Fig. 1) (Creighton, 1993). Originally developed by Lowry *et al.* (1951), this method has undergone many modifications (Fields, 1971; Petersen, 1977; Dunn, 1989). The principal use of this procedure is in biochemistry, although its application in the food industry is increasing. The first application of the Lowry method to determine the concentration of water-soluble peptides in cheese appears to be by Kaminogawa *et al.* (1986) on Gouda-type cheese. The method has since been used to determine protein levels in various HPLC fractions of Cheddar cheese (Cliffe *et al.*, 1989; Bican and Spahni, 1991).

Dye-binding methods

A group of protein quantitation techniques, the dye-binding methods, depend on the reaction of positively-charged amino acid residues in proteins with an acid dye (eg, Amido Black 10B, Acid Orange 12 and Orange G) at ~pH 3 to form an insoluble complex. When using these dyes, the complex precipitates and is easily removed by low-speed centrifugation (2500 *g*), after which the colour of the supernatant is determined



Biuret reaction

$3H_2O.\ P_2O_5.\ 13\ WO_3.\ 5\ MoO_3.\ 10H_2O$

$3H_2O.\ P_2O_5.\ 14\ WO_3.\ 4\ MoO_3.\ 10H_2O$

Active constituents of Folin Ciocalteau reagent: Phosphomolybdic-Tungstic mixed acids

Fig. 1. The Lowry reaction depends on the biuret reaction involving Cu^{2+} binding to peptide bonds and the reaction of tyrosine with Folin-Ciocalteau reagent causing the loss of one, two or three oxygen atoms from the tungstates and molybdates and producing a number of reduced species with characteristic blue colour. This reduction is probably mediated by Cu^{2+}.

spectrophotometrically. The absorbance is inversely proportional to the concentration of proteins/peptides in the solution. Kroger and Weaver (1979) used the binding of Amido Black 10B as an index of proteolysis in cheese. Ashworth (1966), who quantified protein in various dairy products, including ripened cheeses, found that the accuracy of the method for mature cheeses was poor because the dye-binding capacity of cheese peptides decreases on hydrolysis during maturation. For this reason, Kuchroo *et al.* (1983) concluded that dye-binding methods could not be used as an accurate index of proteolysis in cheese. Dye binding methods often result in non-linear results and the dye-binding capacity of different proteins is variable. The use of dye-binding methods to assay peptide levels in cheese has been discussed by Ardo and Meisel (1991). They reiterated the conclusions of Ashworth (1966) and stated that the dye binding capacity of cheese proteins decreased with ripening time.

Bradford method

The method of Bradford (1976) is based on the use of Coomassie Brilliant Blue G250, the absorption maximum of which shifts from 465 nm (rust brown) to 595 nm (blue) when the dye binds to proteins or peptides. Although used extensively in biochemistry, the Bradford method has not been applied to cheese analysis to date.

Erythrosin method

Erythrosin B (a vetinerary drug) forms a stable, intense pink colour when it binds to proteins under acidic conditions (Soedjak, 1994). Erythrosin B has absorption maxima at 517 and 530 nm but on binding to protein, similar to the Bradford method, there is a shift of both absorption maxima to 545 nm. The reaction occurs at room temperature but is significantly faster at 90–95°C when the Erythrosin–protein complex is formed within 2 min. The method is characterised by high sensitivity, good repeatability, limited interference by common reagents and low protein-to-protein variability.

Soedjak (1994) found that the method was as sensitive as the Bradford procedure and more sensitive than the Lowry method. All three procedures were adversely affected by detergents such as Triton X-100, octylglucoside and SDS. This method has not been used to quantify cheese proteins/peptides. Soedjak (1994) reported low protein-to-protein variability and high repeatability when pure solutions of human or bovine serum albumin, avidin, lysozyme, heamoglobin, egg albumin, cytochrome C, gelatin, ribonuelease, α-chymotrypsin, human immunoglobin and horse immunoglobin were analysed using Erythrosin B. This reagent may, therefore, be very useful for determining protein levels in diverse systems such as the water-soluble extract of cheese.

AMINO ACID/AMINO GROUP ANALYSIS BY RAPID METHODS

It is known from studies on different varieties of cheese that methods which quantify free amino groups (both as free amino acids and protein-bound amino groups) generate more information on the extent of proteolysis than methods based on assessment of total nitrogen (Ardo and Meisel, 1991; Gonzalez de Llano *et al.*, 1993). For every peptide bond hydrolysed, a new NH_2 group is formed; therefore, the increase in free NH_2 groups during cheese ripening is proportional to the number of peptide bonds that have been cleaved (Creamer *et al.*, 1985). The free amino groups which are measured using these techniques include groups on both free amino acids and on proteins where they are present both at the amino terminus and as secondary amino groups on amino acids within the protein molecule.

Ninhydrin methods

Ninhydrin reacts with free amino groups to form a purple chromophore [Ruhemann's purple; Ruhemann (1910)] (Fig. 2) with maximum absorbance at 570 nm (Ardo and Meisel, 1991; Fox *et al.*, 1993). Pearce *et al.* (1988) found that the Li-ninhydrin reagent was satisfactory for monitoring proteolysis in ripening cheese. The Cd-ninhydrin assay of Doi *et al.* (1981) was applied

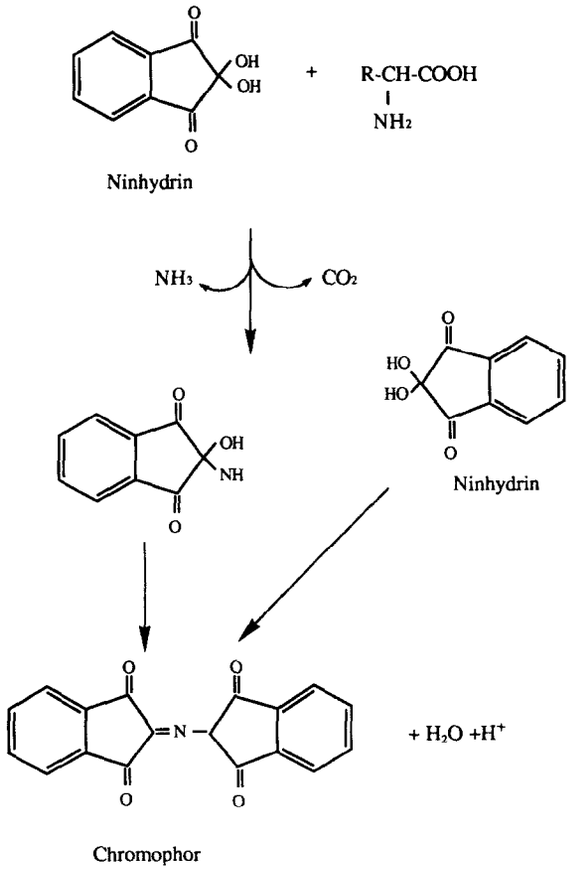


Fig. 2. The ninhydrin reaction.

by Folkertsma and Fox (1992) to assess proteolysis in cheese, and was also used by Law *et al.* (1992) and McSweeney *et al.* (1993). Various fractions of Vacherin Mont d'Or cheese were analysed for amino acid content by Mojarro-Guerra *et al.* (1991) using the ninhydrin assay of Hirs (1967). Gonzalez de Llano *et al.* (1993) found that the Cd-ninhydrin method was a good index of proteolysis and could, in their opinion, replace the macro-Kjeldahl method. Free amino acids can be derivitised with ninhydrin for separation on chromatographic columns (Wilkinson *et al.*, 1992).

Trinitrobenzene sulphonic acid method

The reaction of trinitrobenzene sulphonic acid (TNBS) with primary amines results in the formation of a yellow chromophore which absorbs light maximally at 420 nm (Fig. 3). There are many variations of the TNBS method (Adler-Nissen, 1979; Jarrett *et al.*, 1982) which was introduced by Satake *et al.* (1960) and used by Fields (1971) to quantify amino groups in solutions of proteins, peptides and amino acids. Jarrett *et al.* (1982) found a high correlation between the level of PTA-soluble nitrogen in cheese (measured by the TNBS method) and free amino acids measured by GLC at various ripening times. According to Samples *et al.* (1984), the TNBS method was superior to the Hull procedure for monitoring proteolysis in Cheddar cheese slurries. Kuchroo *et al.* (1983) obtained reproducible results for proteolysis in cheese by reaction with TNBS. They found that the interfering background colour was reduced when pH 4.6- or TCA-soluble fractions, rather than unfractionated cheese, were analysed. Total free amino acids in various cheese types have been estimated by the TNBS method (Clegg *et al.*, 1982; Dunn and Lindsay, 1985; Lemieux *et al.*, 1990; Madkor *et al.*, 1987; Polychroniadou, 1988; Bouton and Grappin, 1994).

o-Phthaldialdehyde method

The fluorimetric *o*-phthaldialdehyde (OPA) method was first described by Roth (1971). In alkaline media containing a reducing agent (e.g. β-mercaptoethanol), OPA reacts strongly with the α-amino group of free amino acids and small peptides (MW < 6000 Da), forming a fluorescent compound (Fig. 4) (Lemieux *et al.*, 1990). OPA adducts also absorb light strongly at 340 nm (Church *et al.*, 1983), allowing spectrophotometric determination of α-amino groups.

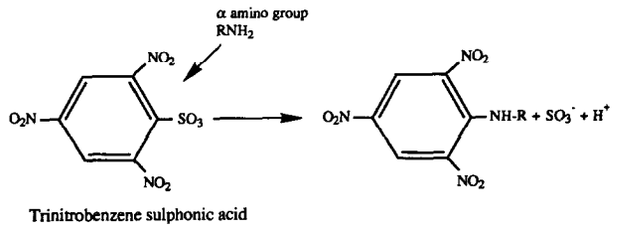


Fig. 3. Reaction of 2, 4, 6-trinitrobenzenesulphonic acid with α-amino groups.

The fluorimetric OPA assay has been used widely to quantify amino acids and proteins (Roth and Hampai, 1973; Viets *et al.*, 1978). Although this method is an order of magnitude more sensitive than the spectrophotometric OPA method, fluoresence quenching by peptide bonds can cause problems (Taylor and Tappel, 1973; Chen *et al.*, 1979). Therefore, in recent years, the spectrophotometric method has become more common (Svedas *et al.*, 1980; Goodno *et al.*, 1981; Rowlett and Murphy, 1981; Church *et al.*, 1983; Lemieux *et al.*, 1990). Frister *et al.* (1989) measured the degree of proteolysis in cheese by monitoring free amino groups in the PTA-soluble fraction. Due to the instability of OPA derivatives, these authors used a modified photometric OPA method involving the addition of *N*,*N*-dimethyl-2-mercaptoethylammonium chloride (DIMAC) which results in stabilising the colour of the chromophor.

The OPA method has been used in dairy research to determine the proteolytic activity of starter (Oberg *et al.*, 1991) and spoilage (Mitchell and Marshall, 1989) microorganisms. The OPA technique is restricted to primary amino groups. Church *et al.* (1983) measured free α-amino groups in the TCA-soluble filtrates of milk. When applied to water-soluble extracts (WSE) of Cheddar, Lemieux *et al.* (1990) found that the OPA and TNBS methods correlated well ($r^2 = 0.98$); although the OPA method was found to be faster and less variable, careful control of the reaction time was necessary.

Analysis of the concentration of free amino acids has been carried out on OPA-derivatives by HPLC (Gonzalez de Llano *et al.*, 1987).

The OPA method has a number of advantages over other protein determination techniques: it is rapid, simple and sensitive and can be applied to all protein types, including milk proteins (Church *et al.*, 1983); the reagent is soluble and stable in aqueous solution and only a single reagent is needed both to stop the action of proteinases (if they are present in solution) and enhance colour development.

Fluorescamine method

Weigele *et al.* (1972) developed a synthetic compound, fluorescamine (4-phenylspiro[furan-2 (3H,1 phthalan)-3,3′dione]) which reacts with primary amines (Fig. 5), producing the same fluorophor generated in the ninhydrin–phenylacetaldehyde reaction, which can be assayed at 480 nm (395 nm excitation). At room temperature, the reaction of fluorescamine is almost instantaneous [half-life of milliseconds (Pearce, 1979)]. The products are highly fluorescent whereas the reagent and its degradation products are non-fluorescent (Undenfriend *et al.*, 1972). The reagent also reacts with water but this

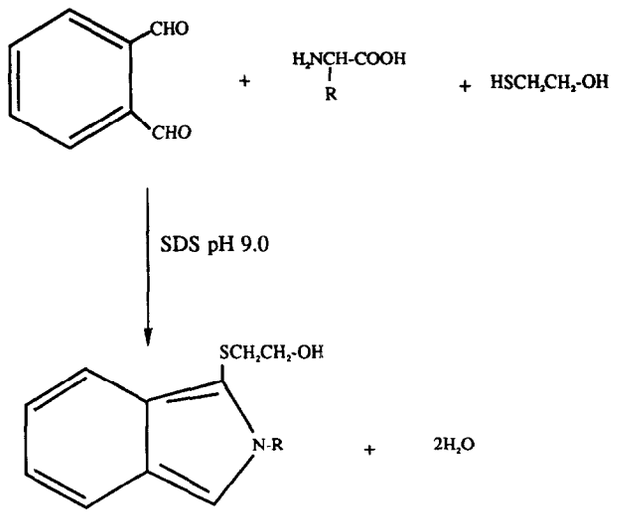


Fig. 4. Reaction of *o*-phthaldialdehyde with a primary amine.

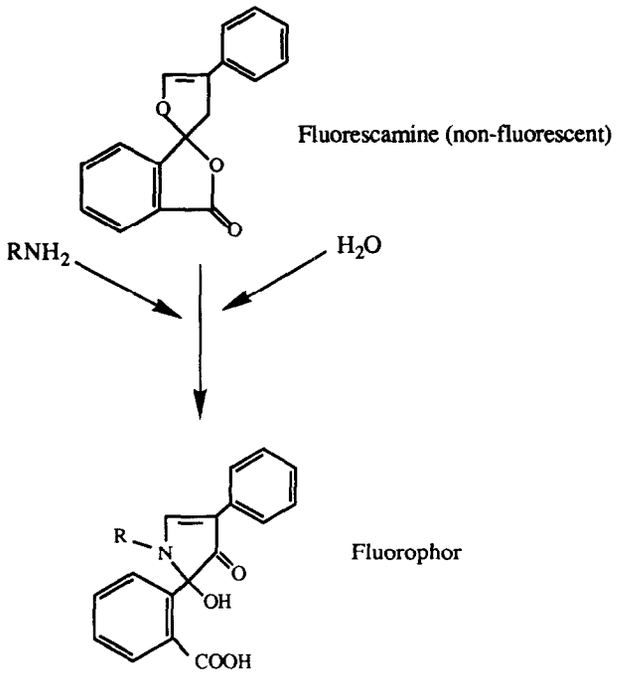


Fig. 5. Reaction of fluorescamine with water and a primary amine.

reaction is much slower than with amines (Undenfriend *et al.*, 1972).

At low fluorescamine concentrations (< 30 mg/100 ml), fluorescence intensity is both concentration and volume dependent (Pearce, 1979). Peptides yield greater fluorescence than their component amino acids and while they fluoresce maximally at pH 7.0, amino acids have maximum fluorescence at pH 9.0 (Undenfriend *et al.*, 1972). Beeby (1980) reported that flourescamine reacts only with α-amino groups at pH 6.0, but reacts with both α- and ϵ-amino groups at pH 8.0. The reduction in fluorescence with decreasing pH, noted by Stein *et al.* (1974), can be explained by the decrease in reactivity of ϵ-amino groups at lower pH values (Beeby, 1980). As well as being pH dependent, the reaction is affected by solvent and reagent composition and the reaction temperature (de Bernardo *et al.*, 1974).

Horowitz (1985) developed a novel flourescamine method to eliminate problems caused by the reaction conditions and fluorescent and non-fluorescent interference. The sample was spotted on a solid support (chromatography paper) and treated with fluorescamine, then photographed (using a KV500 optical filter) and the photographs subjected to fluorescence scanning densitometry. They found this modification particularly useful for processing large numbers of samples from column eluates.

Fluorescamine is more commonly used to assess enzyme activity in solutions than to assess proteolysis in cheese. The method has been used to determine the enzymatic activity of rennet on milk and individual caseins (Pearce, 1979; Chism *et al.*, 1979; Beeby, 1980), the activity of various microbial proteinases (Schwabe, 1973; Rollema *et al.*, 1989) and the formation of TCA-soluble peptides in tryptic digests of various milk proteins. The method has, however, also been applied to the acid-soluble (pH 4.5) fraction of Cheddar cheese (Creamer *et al.*, 1985) and the 12% TCA-soluble fraction of blue (Gomonedo) cheese (Gonzalez de Llano *et al.*, 1993).

The fluorescamine method was found to be superior to the TNBS procedure, giving more consistent results with less scatter (Kwan *et al.*, 1983; Creamer *et al.*, 1985). Schwabe (1973) reported that the fluorescamine method was 100 times more sensitive than the Lowry method.

Rollema *et al.* (1989) used several techniques to monitor the proteolytic activity of various psychrotrophs. While both the fluorescamine and TNBS methods were found to be equally sensitive, high readings in controls (containing no enzymatic activity) for both methods suggested over-estimation of the true values. The authors concluded that results obtained using either of these methods (fluorescamine or TNBS) should be treated with caution.

CONCLUSIONS

Since such a wide variety of techniques for the quantitation of nitrogen compounds are available, researchers must be cautious when choosing the method which best suits their objective. In addition to deciding exactly what is to be quantified (e.g. protein, free amino acids or hydrolysis of peptide bonds), the speed and accuracy required, as well as available equipment, must be considered. Although tedious and time-consuming, the Kjeldahl procedure is probably the best method for measuring total nitrogen in cheese as defatting and protein solubilisation steps required for other procedures may cause loss of protein material. The Kjeldahl method is not suitable for monitoring the cleavage of

peptide bonds. For this purpose, methods using TNBS, Cd-ninhydrin, OPA or flourescamine could be choosen. While many methods could be used to monitor the hydrolysis of κ-casein by rennet, Beeby (1980) pointed out a number of advantages to using the fluorescamine method. In order to monitor the formation of water-soluble nitrogenous compounds in Cheddar cheese, Wallace (1996) found the Lowry method to be accurate, repeatable and to correlate well ($r^2 = 0.971$) with results obtained using the Kjeldahl procedure.

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